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Review

Redesigning the leaving group in nucleic acid polymerization

P. Herdewijn [a,*], P. Marlière [b]

[a] Laboratory of Medicinal Chemistry, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium
[b] Isthmus SARL, 13 rue Saint-Amand, F-75015 Paris, France

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ABSTRACT

Artificial nucleic acids have the potential to propagate genetic information in vivo purposefully insulated from the canonical replication and transcription processes of cells. Natural nucleic acids are synthesized using nucleoside triphosphates as building blocks and polymerases as catalysts, pyrophosphate functioning as the universal leaving group for DNA and RNA biosynthesis. In order to avoid entanglement between the propagation of artificial nucleic acids in vivo and the cellular information processes, we promote the biosynthesis of natural and xenobiotic nucleic acids (XNA) dependent on the involvement of leaving groups distinct from pyrophosphate. The feasibility of such radically novel biochemical systems relies on the systematic exploration of the chemical diversity of nucleic acid leaving groups that can undergo the catalytic mechanism of phosphotransfer in nucleic acid polymerization. Initial forays in this research area demonstrate the wide acceptance of polymerases and augur well for in vivo implementation and integration with canonical metabolism.

1. Introduction

The chemical constitution of biopolymers, found in living organisms, results from the diversity not only of the 'building blocks' i.e. the monomers present in the macromolecules once synthesized, but also from the diversity of the leaving groups used for activating the monomers (which are absent from the biopolymers and released during the synthetic process). This 'backstage scenery' of biosynthesis has so far not been subjected to a systematic study to define the potential for evolving artificial biodiversity and alternative bioprocesses. In the case of nucleic acids, pyrophosphate (PPi) is a universal leaving group that is released from nucleoside and deoxynucleoside triphosphates. No exception to the usage of pyrophosphate as leaving group has ever been reported to occur in any natural cell or virus. This situation contrasts dramatically with protein biosynthesis, where leaving groups occur as informational molecules, in the form of tRNAs, whose sequences vary in a cognate fashion with the chemical structure of the activated amino acids. Here, we explore the design principles of biochemical leaving groups, review the experimental attempts at diversifying leaving groups in nucleic acid biosynthesis in vitro and propose scenarios for introducing such alternative leaving groups in vivo.

Introduction of alternative leaving groups (ALG) distinct from pyrophosphate pertains to both the directed evolution of the natural nucleic acids RNA and DNA, and to the implementation of an additional category of nucleic acid in vivo i.e. XNA. The various configurations to be tackled from wild type (micro)organisms are summarized in Table 1.

2. Leaving group (bio) chemistry

Heterolytic cleavage of a molecule gives rise to two fragments, one of them is called the leaving group. When this leaving group takes away an electron pair it is called nucleofugal. This process usually occurs in the presence of an attacking reagent, in this case a nucleophile. The best leaving groups are the weakest bases, and protonation increases leaving group potential. This is a very common reaction in organic chemistry which is usually carried out in organic solvents. This reaction is also very common in biology

Abbreviations: 3-phosphono-L-Ala, 3-phosphono-L-alanine; ALG, alternative leaving groups; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CoASH, coenzyme A; dCMP, deoxycytidine monophosphate; dGMP, deoxyguanosine monophosphate; DNA, deoxyribonucleic acid; dNTP, deoxynucleotide triphosphate; dTMP, thymidine monophosphate; dTTP, thymidine triphosphate; HIV-1, human immunodeficiency virus-type 1; IDA, iminodiacetate; ILA-dAMP, β-imidazole lactic acid-dAMP; KF, Klenow fragment; Km, Michaelis constant; L-Asp, L-aspartic acid; L-Glu, L-glutamic acid; L-His, L-histidine; PAP, phosphoadenosine phosphate; PPi, pyrophosphate; RNA, ribonucleic acid; RT, reverse transcriptase; TPI, triphosphoiminodicarboxylate; tRNA, transfer-RNA; UDP, uridine diphosphate; V_{max}, maximum rate; XNA, xenobiotic nucleic acids

* Corresponding author. Fax: +32 16 337340.
E-mail addresses: piet.herdewijn@rega.kuleuven.be (P. Herdewijn), phmarliere@gmail.com (P. Marlière).

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Table 1
Stepwise reassignment of leaving groups to canonical (DNA, RNA) and xenobiotic (XNA) nucleic acids in genetically reprogrammed living organisms.

Class of organism	RNA	DNA	XNA
0 (wild type)	PPi	PPi	–
I	ALG	PPi	–
II	PPi	ALG	–
III	PPi	PPi	PPi
IV	PPi	PPi	ALG

where it has to be performed in water, restricting the choice of leaving groups that can be used, for stability and solubility reasons. Several leaving groups are used in biology such as thiols (CoASAc), dialkyl sulphides (S-adenosyl methionin), enolethers (phosphoenolpyruvate), cyclic ureas (carboxylated biotin), but in most cases it is a phosphate or a pyrophosphate group (for example in UDP-glucuronic acid, 3′-phosphoadenosine-5′-phosphosulfate, ATP). UDP, for example, is the most common leaving group in polysaccharide synthesis. In some cases the leaving group may be a large molecule like tRNA in peptide synthesis. In all events, however, the leaving group itself is a common metabolite (CoASH, S-adenosyl homocysteine, pyruvate, biotin, UDP, PAP, ADP, AMP and so on) that can be reused or further processed. The example of protein synthesis is interesting, because it is an example where the leaving group (tRNA) covers information. This is necessary because during protein synthesis information needs to be translated from one biopolymer (RNA) to another (protein). tRNA is, likewise, considered as a crucial molecule in the origin of life and one can ask the question if the principle of putting information in the leaving group is not a general approach to exponential growth and diversification of information [1]. Another example of information transfer during polymerization is that of DNA and RNA synthesis. In this case, however, there is a direct readout of information and pyrophosphate functions as the leaving group. These polymers (nucleic acids and proteins) are central to biological studies and their properties and functions have been studied intensively. However, not much attention has been given to the potential role of the leaving group in metabolism, information and energy transfer. These issues become important when considering the use of artificial nucleic acids for storing information in a cell to be used for defined purposes such as the production of therapeutics or coding for a polymer indispensable for the survival of the cell.

3. Enzymatic nucleic acid synthesis

For developing artificial genes based on synthetic nucleic acids, one has to realize that in a living cell, many functions are carried out by similar molecules. The numbers of chemical principles that are used within a cell are limited. The most striking example is that of nucleoside triphosphates, characterized by the presence of high energy phosphoanhydride bonds that drive their chemistry in a biological context. ATP is involved in information storage and transfer (ATP as a building block for RNA), in energy transfer (ATP as an energy carrier to combine molecules), in regulatory functions (at the level of kinases and signal transduction) and in driving metabolic pathways (ATP as an activated carrier molecule). This multifunctionality of nucleoside triphosphates in general, can be a dangerous trap when trying to replace the natural genetic system by an artificial 'biopolymer' for defined purposes. Therefore it is important to dissect the role of nucleoside triphosphates in building the information system, from their role in other cellular functions (energy regulation, metabolism**),** by means of chemical modification. For safety reasons as well as efficiency reasons, the cellular chassis should function independently from the engineered device.

DNA and RNA are synthesized from nucleoside triphosphates. In this process, one nucleotide is attached (via its 5′-phosphate) to the 3′-hyroxyl group of the growing nucleic acid chain, using pyrophosphate as leaving group. The pyrophosphate is further hydrolyzed to release two phosphate molecules. The nucleoside triphosphates themselves are synthesized from the monophosphates through the action of nucleotide kinases and nucleoside diphosphate kinases. Because of the many functions of nucleoside triphosphates, there is ample room for interference with biological processes when triphosphates of modified nucleosides are brought into the cell (a process that is needed for the propagation of artificial nucleic acids in vivo). Indeed, it would be difficult to install additional nucleoside triphosphates without interfering with DNA and RNA metabolism, cell energy supply or substrate-level phosphorylation.

One way to avoid this intricacy is to use different types of building blocks for the enzymatic synthesis of the natural nucleic acids and for the enzymatic synthesis of the artificial nucleic acids. This can be realized by substituting the pyrophosphate moiety with alternative leaving groups in the precursors for the synthesis of artificial information systems. Indeed, the chemical structure of the leaving group can be elaborated leading to segregation of the polymerization of XNA from that of DNA and RNA and establishing an informational enclave. Another result, as a consequence, is disentangling the XNA polymerization from the phosphoanhydride economy of the cell and thus establishing an energetic enclave as well. In this case, these leaving-group-modified-nucleotides should not be accepted as substrate by regular natural polymerases and vice versa (the evolved polymerase should not accept natural nucleoside triphosphates as building blocks). The use of alternative leaving groups would result in genetic enclaves with metabolic independence, without having to physically separate precursors of the artificial nucleic acids from those of DNA and RNA. Indeed, the implementation of novel leaving groups in metabolism could overcome the need for physical compartmentalization of unnatural information transfers and enable the launching and sustaining of autonomous hereditary procedures in vivo.

The selection of new leaving groups for the enzymatic synthesis of nucleic acids should be accompanied by a selection of appropriate polymerases, which can use the modified nucleotides as building blocks for gene synthesis independent of the cellular gene-synthesis machinery. The activated nucleotides with alternative leaving groups should, likewise, not be recognized by other enzymes involved in cellular functions where nucleoside triphosphates play a role. Given the different biochemical mechanisms involved in polymerization, energy supply and metabolic regulations, this should be within reach. Another prerequisite of an alternative leaving group is that it is non-toxic. If it corresponds or could be converted to a common metabolite, it could fit into recycling pathways, and the accumulation of toxic by-products of the polymerization process could be prevented.

4. Building blocks for XNA synthesis

The ideal properties for such a nucleotide analogue (in vitro and in vivo) would be: water soluble, chemically and enzymatically stable, recognized by the active site of polymerases and serve as substrate for the polymerase; incorporation into the growing nucleic acid should be a mechanistic-based approach, chain elongation should occur, the leaving group should be non-toxic (for example a common cellular metabolite), and the leaving group should be actively degraded or recycled to make the polymerization process irreversible. This mechanistic-based approach is an important issue as it would imply that a relatively stable molecule can be used that is converted into a reactive transition state once

bound in the active site of the polymerase. It will be necessary to find a subtle equilibrium between the potential for enzyme-catalyzed incorporation into nucleic acids and chain elongation together with the kinetics of these reactions, the enzymatic and chemical stability of the molecules and the fidelity of the base-pairing rules.

Many reviews have summarized the different DNA-polymerase families. DNA-dependant DNA-polymerases are subdivided into six families by sequence homology. However, the polymerization domains of all polymerases share a common architecture and the general mechanism is similar including: (a) binding DNA substrate; (b) binding nucleoside triphosphate; (c) conformational change to form an activated complex; (d) primer extension by phosphodiester bond formation; (e) conformational change accompanied by the release of pyrophosphate; (f) translocation of the enzyme to the next base in the template, as summarized by Holmberg et al. [2]. Divalent metal ions (typically Mg^{2+}), coordinated between carboxylate moieties of the enzyme and phosphate functions of the nucleotides, are involved in the catalytic process of phosphodiester bond formation between the 3′-OH of the primer and the α-phosphate group of the incoming nucleotide. Because of this similar mechanism, it can be expected that the approach may be useful for several polymerases, although initial experiments show considerable differences between HIV-RT, Terminator DNA polymerase, Taq, Vent (exo-) and KF (exo-) DNA polymerases. However, Since HIV-1 RT shows broad substrate-specificity, this enzyme was chosen by us as a primary polymerase for selection of potential leaving groups as potential pyrophosphate mimics.

The molecular mechanism of the polymerization reaction and the structural changes involved seems to be conserved between different polymerases [3]. The active site pocket is created by conformational changes occurring after binding of the nucleoside triphosphate. In the case of RT, binding of a dNTP induces substantial side chain repositioning in the active site, resulting in a contact between Lys65 and Asp72 with the incoming dNTP (with the γ- and α- phosphate, respectively) and involving the carboxylate of Asp110 in chelation with the magnesium ion of the incoming dNTP [3]. However, the interaction between Lys65 residue of RT and the γ-phosphate of the incoming nucleotide is not absolutely required for the nucleotidyl transfer reaction [4]. Nucleoside diphosphates may therefore be utilized as substrate of RT in a single nucleotide incorporation experiment, but the efficiency is very low (k_m of dADP is 9.9 μM versus 0.02 μM for dATP, k_{cat} of dADP is 0.02 min-1 versus 2.3 min-1 for dATP, a reduction in efficiency (k_{cat}/k_m) by a factor of 50 000). Dinucleoside pyrophosphate can also be used as substrate for polymerases, likewise with reduced efficiency [1]. The nucleotidyl transfer seems to be a general acid catalyzed reaction in which active-site amino acid residues are involved in the protonation of the pyrophosphate leaving group [5]. Two metal ions are responsible for lowering the pK_a of the primer 3′-OH group, for orientation of the nucleoside triphosphate, for the stabilization of the pentavalent transition state and for the release of pyrophosphate [5]. During a cycle of single nucleotide incorporation two enzyme conformations exist. The translocation itself is induced by the dissociation of pyrophosphate (released by the phosphoryl transfer reaction) after the nucleotide addition, at least for T7 RNA polymerase [6]. The conformational changes induced by the pyrophosphate release prevents the 3′ terminus from binding to the N site (product binding), such that only the P (primer binding) site is available for binding the primer terminus [6]. The contribution of the pyrophosphate leaving group to DNA synthesis, in terms of catalytic efficiency and fidelity of DNA polymerase β, has been studied. These data suggest a leaving-group-induced change in the rate limiting step due to stabilization of pyrophosphate elimination [7]. Chemical modification of the leaving group influences active-site structural differences between the correct and incorrect base-paired transition state [8].

Knowing the complexity of the polymerization process, we expected that it would be a difficult task to discover new leaving groups with similar properties to pyrophosphate.

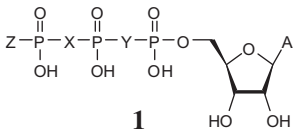
As a result, modification of the triphosphate moiety of natural nucleosides with the aim of discovering alternative substrates for polymerases is a research area that is not well documented. The reason for the fact that research on triphosphate modification of nucleosides for the enzyme catalyzed polymerization is underdeveloped is that there is a general belief that it will be difficult to find a chemical substitute for the pyrophosphate moiety of a nucleoside triphosphate that has similar leaving group capabilities, water solubility, and high energy content while still being recognized by polymerase enzymes.

Generally, such modifications have been investigated in searching for inhibitors of enzymes such as adenylate cyclase and reverse transcriptase or as inhibitors of nucleotide binding receptors. Research on the substrate specificity of polymerases is mainly focused on modifications in the base moiety or the sugar moiety of the nucleotides, but seldom in the triphosphate moiety. The enzymatic synthesis of modified nucleic acids (phosphoramidates) using sugar modified nucleoside triphosphates was pioneered by Letsinger [9] using d_NTP as substrate and DNA polymerase I of *Escherichia coli* as catalyst. The aim of this research is to synthesize backbone modified nucleic acids using an enzymatic approach. Most information about sugar- and triphosphate- modified nucleosides and their incorporation into DNA using enzymes (reverse transcriptase) is available from the HIV literature (with the goal of finding potent inhibitors of HIV replication) [10].

The modifications that have been investigated so far are mainly minor modifications within the triphosphate moiety itself. The most studied groups have been methylene phosphonate (**1**a–c), phosphoramidate (1d, 1g) and thiophosphate analogues of ATP (2α-c) (Scheme 1, [11–25]). Investigations on the structure of imidodiphosphate and methylene diphosphoric acid [26] in comparison with pyrophosphate reveals that the P···P distance is similar in these three compounds. An interesting observation is that the imidodiphosphate linkage occurs naturally [27].

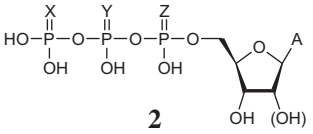
The β,γ-methylene analogue (1b) is able to substitute for ATP in an RNA polymerase reaction [28]. Taq polymerase is able to incorporate the Sp-diastereomer of dNTPαS into DNA [29]. ATPαS can be used as substrate by DNA dependent RNA polymerase from *E. coli* [30]. Also, modifications at the terminal (γ) phosphate of the nucleoside triphosphate (1e–g) seem to be well accepted by polymerases. For example the fluoro [18], OMe [19] and anilidate [19] modified ATP's (scheme 1) are substrates for DNA-dependent RNA polymerase of *E. coli*. 5′-P-Borane substituted nucleoside triphosphates (3a) are excellent substrates for Sequence (a modified T7 DNA polymerase) [20].

The [α,β-imino] triphosphate analogue of ATP is an effective substrate for T7 RNA polymerase [31]. The β,γ-difluoromethylene analogue of ATP (1 h) is an improved substrate for *E. coli* DNA-dependent RNA polymerase (relative to the methylene analogue) [21] (Scheme 1). The modification of the leaving group in the β,γ position, however, alters efficiency and fidelity of the incorporation reaction using DNA polymerase β [7]. Other phosphonate analogues that have been found to be substrates for polymerases are phosphonomethoxy's (1i) [22] and P^{α}-alkyl phosphonates (3b) [23–25]. The first compound was tested using bacterial DNA-dependent RNA polymerase, while the second type of compounds were evaluated as substrates for terminal transferase, HIV-RT, AMV-RT and DNA polymerase β.



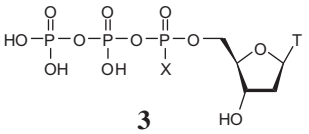
1

	Z	X	Y	Ref
a)	OH	O	CH_2	[11] [12]
b)	OH	CH_2	O	[13]
c)	OH	CH_2	CH_2	[14]
d)	OH	NH	O	[12]
e)	F	O	O	[18]
f)	OMe	O	O	[19]
g)	HNPh	O	O	[19]
h)	OH	CF_2	O	[21]
i)	OH	CH_2O	O	[22]



2

	X	Y	Z	Ref
a)	S	O	O	[15]
b)	O	S	O	[16]
c)	O	O	S	[17]



3

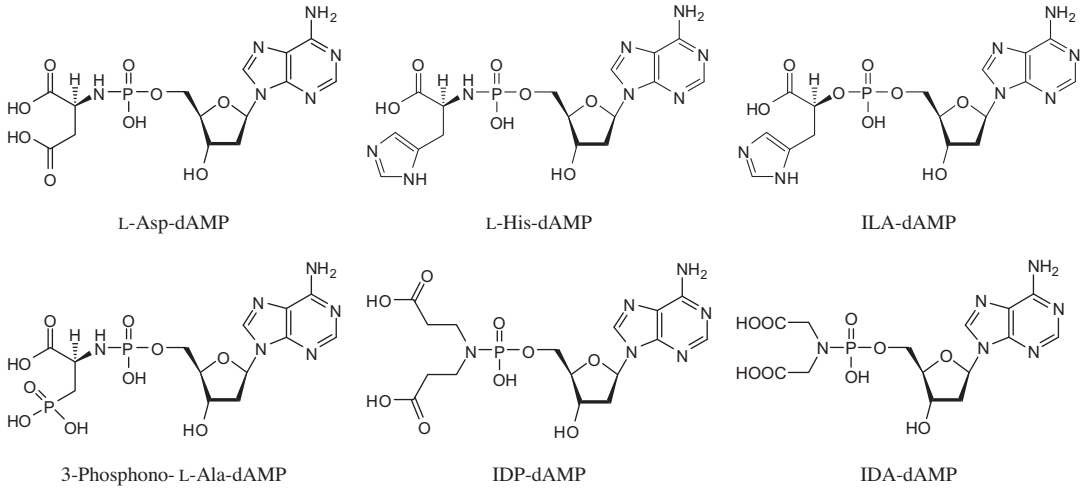
	X	Ref
a)	$^{-}BH_3$	[20]
b)	CH_3, $C_{10}H_{21}$	[23-25]

Scheme 1. Structure of nucleoside triphosphates, chemically modified in the triphosphate moiety.

5. Metabolic accessible leaving groups

One of the prerequisites for selecting a new leaving group for the enzymatic polymerization of xeno nucleic acids is that the potential leaving group must be metabolically accessible. Three levels of metabolic accessibility can be considered e.g. a leaving group that is a common metabolite in all cells (e.g. L-Asp), a leaving group that is produced by nature in a selected series of organisms (e.g. phosphono-L-Ala), a leaving group for which a metabolic scheme can be easily designed based on well-known metabolic reactions occurring in micro-organisms (e.g. iminodiacetate). We have elaborated several examples of these three groups.

The first example where the structure of the leaving group of the nucleoside triphosphate mimic deviates considerably from that of the natural pyrophosphate moiety while keeping the capacity to be recognized by polymerases (in casu reverse transcriptase), is that of L-Asp-dAMP and L-His-dAMP [32] (Scheme 2). It seems that L-aspartic acid and L-histidine provide the proper geometric and spatial arrangement of functional groups for the formation of a tertiary complex in the active site of the enzyme. In addition, the binding of L-Asp-dAMP or L-His-dAMP and catalytic metal ions allows further rearrangements of the catalytic amino acid residues and positioning of the 3′-OH group of the terminal nucleotide of the primer, to make the phosphodiester bond formation possible by cleavage of the αP-N bond. Likewise, L-Asp-dGMP, L-Asp-dCMP and L-Asp-dTMP are substrates for HIV-RT, while retaining canonical base-pair selectivity [33]. Incorporation of L-His-dAMP by HIV-1 RT however proceeded with lower efficiency than with L-Asp-dAMP. However in contrast to its congener L-Asp-dAMP, primer chain elongation was slightly improved when L-His-dAMP was used. It is important to note that regardless of the phosphoramidate mimic, DNA synthesis dramatically slowed down after incorporation of two nucleotides, for which the biochemical reason is not clear.



Scheme 2. Representative examples of nucleotides with alternative leaving groups.

Besides HIV-RT, the terminator DNA polymerase is also able to accept L-Asp-dAMP as substrate and to elongate the DNA chain by several nucleotides [34]. On the other hand, glutamate, glycine, alanine, proline, tyrosine and serine, or the methyl ester of aspartate and histidine were poor substrates for the enzyme. The fact that L-Glu-dAMP is not recognized as substrate, while L-Asp-dAMP is recognized, means that the process of recognition and incorporation is very specific. Encouraging results, however, are: that the primer can be extended with up to six deoxyadenosines (although polymerization starts to stall after incorporation of two to three nucleotides), that the incorporation reaction follows Watson- Crick rules and is stereoselective (L- versus D-amino acids), and that the incorporation of the four building blocks L-Asp-dAMP, L-Asp-dCMP, L-Asp-dGMP, L-Asp-dTMP) follows Michaelis–Menten kinetics.

An intriguing question is how these alternative leaving groups are bound in the active site and what the activation mechanism is. It is difficult to rationally design binding modes for alternative substrates in the active site of an enzyme while keeping its activation mechanism for the reaction close to the natural system. Initial molecular modelling experiments have demonstrated that L-Asp-dAMP and L-His-dAMP and dTTP might bind in a different way in the active site of RT, but that similar amino acids are involved (Arg 72, Lys 65, Asp 110, Asp 185, Asp 186). Both magnesium ions play a role in the binding of the three substrates. Steady state kinetic analysis of L-Asp-dAMP incorporation indicates that V_{max} for incorporation of L-Asp-dAMP in DNA is only threefold lower than that of an HIV RT natural substrate. The high k_m value for L-Asp-dAMP implies weak binding to the polymerase active site. This suggests fast and efficient nucleophilic displacement of the amino acid moiety once the amino acid phosphoramidate substrate is bound in the active site and formation of a phosphodiester bond has occurred. The specificity, or a V_{max}/k_m value, for insertion of amino acids phosphoramidates versus a natural substrate is decreased 1300-fold. Similar kinetics is observed for L-Asp-dCMP, L-Asp-dGMP and L-Asp-dTTP (Table 2). It should be mentioned, however, that measuring kinetics based on percent gel band extension per minute gives mostly results for single nucleotide incorporation that are underestimated, because in this experiment the whole process is monitored (including conformational changes and DNA release) [35].

These encouraging results motivated us to further explore new potential metabolic accessible leaving groups for enzyme-catalyzed DNA synthesis. *N*-Methyl-L-aspartic acid is as good a leaving group as L-aspartic acid but with lower base-pair selectivity (A against C and A against A incorporation) [33]. Efficient incorporation and chain elongation was also observed for the δ-dicarboxybutyl amine leaving group, although the full potential of this system could not be evaluated because only the cytosine nucleotide was available (which requires a poly-G template) [36].

The superior leaving group properties of a phosphodiester (versus a phosphoramidate) was demonstrated by comparing several O- and N- linked dAMP analogues. The leaving group efficiency increases in the order β-alanine< β-hydroxypropionic acid < glycine < α-hydroxyacetic acid [37]. When comparing β-imidazole lactic acid-dAMP (ILA-dAMP) and L-His-dAMP, the former is a better substrate. The substrate affinity is doubled and the maximum velocity is increased by a factor of 10 (Table 3). However, pausing is also observed here after incorporation of two to three nucleotides, indicating that this effect is not caused by the nature of the linker (P–O versus P–N) [38].

The influence of electrostatics has been evaluated by substituting the β-carboxylic acid of L-Asp-dAMP with a phosphonate group, giving 3-phosphono-L-Ala-dAMP (Scheme 2) [39]. This is a considerably improved substrate, showing a V_{max} which is only 1.3-fold lower than that of dATP and a k_m that is 78-fold higher (Table 4). The V_{max}/k_m ratio is 99 times lower than for dATP. When compared with L-Asp-dAMP, improved chain elongation has been observed, but is still less effective than with dATP. The phosphonate group and the phosphoramidate group seem to mimic the γ- and α-phosphate groups of deoxynucleotide triphosphates, respectively. The carboxyl group functions as a mimic of the β-phosphate group. Among the four bases studied (A, C, T, C), the A, G and T analogues are good substrates, but C is only incorporated at higher concentrations. The efficiency of the leaving group seems to be base-dependent and the Watson–Crick base pairing rules are respected. The base-dependent effect is not clear, but it is consistent with the observation that the processive DNA systems using dTTP are less efficient than using dATP. Stacking interactions may be involved here [40]. Apparently, 3-phosphono-L-alanine may replace pyrophosphate in its elongation function, but less efficiently as is clear from the lower processivity of DNA synthesis. However, when comparing the efficiency of using 3-phosphono-L-Ala-dNMP (N = A, T, G) as substrates for different enzymes [HIV-RT, Vent(exo$^-$), Therminator polymerase] the DNA-dependent DNA polymerase activity is significantly higher for Vent(exo$^-$) polymerase than for HIV-1 RT, and Vent(exo$^-$) polymerase is more accurate than Therminator polymerase, based on Watson–Crick pairing. Vent(exo$^-$) polymerase may synthesize DNA efficiently without stalling effects. The stalling effect seems to be polymerase dependent and, likewise, the optimal pyrophosphate mimic for the enzyme catalyzed synthesis of DNA is polymerase dependent [41].

Since we aim to develop cellular systems using orthogonal nucleic acids as information systems in bacterial organisms, it would be important that the above mentioned modified nucleotides are accepted as substrate by selected polymerases of bacterial origin. PolIII belongs to polymerase family C and it is found in the replisome of *E. coli* at a low density of molecules [42]. Its α-subunit, which is endowed with polymerase activity, accepts L-Asp-dAMP as substrate for both single nucleotide incorporation and for chain elongation. (A. Giraut et al., unpublished results) This polymerase seems to be an ideal candidate for directed evolution for implanting an orthogonal information system in vivo.

As demonstrated above, the common metabolite aspartate coupled to the phosphate group of deoxynucleotides by a P–N phosphoramidate bond provides unnatural adducts acting as competent substrates in the HIV-1 RT directed template-dependent DNA polymerisation. Through broadening our search of the chemical space, we addressed the synthesis and enzymatic assay of the deoxyadenylate adduct of iminodiacetate [43] (Scheme 2).

Table 2
Steady-state kinetics of the incorporation of L-Asp-nucleotides in DNA catalyzed by HIV-RT (polymerase at 0.015 V/μL).

Substrate	V_{max} [pmol min^{-1} U]	K_M [μM]	V_{max}/K_M ($\times 10^6$)
dATP	8.39 ± 0.82	0.46 ± 0.15	18.1
L-Asp-dAMP	2.63 ± 0.13	185.3 ± 24.55	0.014
dGTP	28.81 ± 1.55	0.54 ± 0.15	53.4
L-Asp-dGMP	2.14 ± 0.13	168.8 ± 27.9	0.013
dTTP	30.82 ± 1.44	0.53 ± 0.07	58.2
L-Asp-dTMP	2.33 ± 0.22	288.2 ± 61.72	0.008
dCTP	5.62 ± 0.80	3.74 ± 0.95	1.5
L-Asp-CMP	0.59 ± 0.04	130.8 ± 19.8	0.005

Table 3
Kinetic parameters of the incorporation of dAMP into DNA by HIV-1 RT. Polymerase at 0.0063 U/μL (dATP and ILA-dAMP) or 0.025 U/μL (L-His-dAMP).

Substrate	V_{max} [pmol min^{-1} U]	K_M [μM]	V_{max}/K_M ($\times 10^6$)
dATP	5.114 ± 0.397	0.48 ± 0.13	10.65
L-His-dAMP	0.330 ± 0.033	505.0 ± 114.8	0.0007
ILA-dAMP	2.746 ± 0.144	204.7 ± 30.8	0.013

Table 4
Steady-state kinetics of single nucleotide incorporation into DNA by HIV-1 RT at 0.0125 U/μL.

Substrate	V_{max} (nM/min)	K_m (μM)	V_{max}/K_m ($\times 10^{-3}$ min^{-1})
dATP	12.5 ± 0.26	1.01 ± 0.11	12.37
3-Phosphono-L-Ala-dAMP	9.84 ± 0.26	78.9 ± 6.70	0.125

By selecting iminodiacetate as leaving group, we altered the substitution pattern of the nitrogen atom of the phosphoramidate moiety, while taking care of potential chelating properties and staying focused on potential metabolic accessibility of the new leaving group. Enhanced coordination of the magnesium ion by the leaving group moiety of the modified nucleotide might influence incorporation kinetics and efficiency of the chain elongation reaction. In addition, iminodiacetic acid can be considered as potentially prone to catabolism into non-toxic cellular constituents (i.e. glycine and a two-carbon atom fragment). As a consequence, this degradation reaction may have an effect on the equilibrium of the nucleotide incorporation reaction. The iminodiacetate dAMP phosphoramidate (IDA-dAMP) possesses no chiral centre (ensuring less stereochemical constraint than for the chiral L-Aspartic acid).

Along with showing better elongation results, IDA-dAMP also demonstrates somewhat better incorporation kinetics than L-Asp-dAMP for HIV-1 RT (Table 5). The measured V_{Max}/K_M ratio towards HIV-1 RT is 3 orders of magnitude lower than that of dATP. Similarly to the L-Asp phosphoramidate analogue, the K_M of IDA-dAMP is much higher than for the natural substrate, whereas the V_{Max} is 1.8-fold lower. The increased capacity for chain elongation is paralleled by a similar increase in incorporation efficiency. It is clear that enzyme evolution should focus on the selection of a polymerase enhancing K_M (rather than V_{Max}) which is easier to achieve than the inverse.

The observed full-length elongation obtained when using IDA-dAMP and a purine-elongated template, could be due to the increased affinity of the longer template overhang for the enzyme and the formation of a more stable tertiary complex. The template length seems to influence the ability of HIV-1 RT to recognise and incorporate an incoming dNTP carrying a modified leaving group. It has been shown by Kohlstaedt in 1992 that the template strand binds to the fingers subdomain of the polymerase active site [44]. In addition, the binding affinity of RT with DNA duplexes as template is increased when template overhang was extended by 6 additional bases [45]. From models based on crystal structures of the complex [45,46], the β3-β4 loop situated in this domain contacts the template three nucleotides upstream from the primer terminus (near or at Leu74). Kew et al. postulated that a stronger complex between the fingers subdomain of a HIV-RT and the template overhang may be responsible for higher processivity by the enzyme [47]. Another explanation for a decrease in elongation efficiency when using unusual substrates is that the pausing and strand terminating effect can be sequence selective [48] and/or by the absence of the energetically rewarding hydrolysis of PPi which could drive the polymerization reaction [49]. Nevertheless, the appearance of a pausing effect is dependent on the structure of the leaving group, on the polymerase, and on the concentration used in the experimental set-up.

Modelling experiments showed us that in the ground state the carboxylate groups of IDA-dAMP are most likely to be bound to an intricate network of amino acid residues located in the enzyme's active site via divalent cations. This model implies that the stabilisation of IDA-dAMP is enhanced and involves 6 amino acid residues instead of 4 with the natural substrate and 5 with L-Asp-dAMP. This increase in amino acid interactions observed in the complex formation with IDA-dAMP is reflected in a change in K_M. The electrostatic involvement of residues Gln155A and Asp186A has not been observed in previous studies. The difference in binding affinity between pyrophosphate and iminodiacetic acid is likely to contribute to a difference in kinetics [50].

By synthesizing and evaluating a series of IDA-dAMP analogues, either bearing an extended aliphatic chain in the amino acid function, or a phosphonic acid moiety, we observed that iminodipropionic acid is a leaving group that shows improved kinetics and elongation ability compared to IDA-dAMP (Table 5) [51]. When using HIV-1 RT as catalyst, the V_{max} of IDP-dAMP is similar to the natural substrate. However, an increase in the K_M value for the modified substrate is observed, compared to dAMP. The catalytic efficiency of IDP-dAMP toward HIV-1 RT is therefore only reduced by 83-fold compared to dAMP.

6. Metabolic accessibility and recycling

A constraint of crucial importance when reprogramming biopolymers using unnatural building blocks lies in the import of xenobiotic molecules into living cells. Passive diffusion would be insufficient for feeding the biosynthetic flux and active transport has to be mobilized for this purpose. Five experimental scenarios can be contemplated according to availability of metabolic enzymes and uptake systems (Table 6). In the simplest scenario (A) the molecules each consisting in a xeno nucleotide activated with the same alternative leaving group are supplied exogenously to the cells and transported in the cytoplasm to serve as substrates of a xeno polymerase. This has the advantage of not involving metabolic enzymes, but implies the discovery or evolution of at least one transport catalyst, and a heavy burden for the chemically synthesizing complex and fragile activated xeno nucleotides. In the opposite scenario (E) all xeno nucleotides activated with the alternative leaving group are synthesized endogenously by reprogrammed cells. This entails the daunting task of evolving numerous metabolic enzymes. Moreover, it would also potentially create a biohazard, since chemically autonomous cells able to propagate xeno nucleic acids could be suspected of seeking niches in natural habitats.

The three remaining scenarios (B, C, D) (see Table 6) potentially present the least experimental drawbacks and environmental hazards. Accordingly activated xeno nucleotides would be synthesized by at least one metabolic enzyme, but either the corresponding

Table 5
Determination of the kinetic parameters of the incorporation of dAMP with the natural pyrophosphate leaving group (dATP) the iminodiacetate leaving group (IDA-dAMP) and the iminodipropionate leaving group into DNA by HIV-1 RT (polymerase at 0.025 U/μL[a] and at 0.0125 U/μL[b]).

	V_{max} [nM min^{-1}]	K_M [μM]	V_{max}/K_M ($\times 10^{-3}$ min^{-1})
dATP[a]	24.5 ± 1.1	0.44 ± 0.1	55.7
IDA-dAMP[a]	13.4 ± 1.4	226 ± 61	0.06
dATP[b]	12.4 ± 0.4	0.93 ± 0.1	13.3
IDP-dAMP[b]	11.8 ± 0.5	73.7 ± 9	0.16

Table 6
Scenarios of nutritional dependency and metabolic capability in using alternative nucleic acid leaving groups.

Scenario	Nucleos(t)ide	Alternative leaving group	Activated nucleotide
A	–	–	Uptake
B	Uptake	Uptake	Biosynthesis
C	Biosynthesis	Uptake	Biosynthesis
D	Uptake	Biosynthesis	Biosynthesis
E	Biosynthesis	Biosynthesis	Biosynthesis

xeno nucleosides or the alternative leaving group would have to be supplied exogenously to reprogrammed cells.

The use of natural amino acids such as aspartate or histidine as alternative nucleic acids leaving groups are compatible with scenarios A, D and E, but since the latter scenario must be avoided, these amino acids should be conjugated to artificial nucleotides that cannot form in known metabolic pathways (scenarios A, B and D, excluding C and E).

Phosphonoalanine, which does not occur in canonical metabolism, could serve in scenarios A–D. However, it occurs naturally in the sea anemone *Zoanthus sociatus* [52] and the protozoan *Tetrahymena pyriformis* [53] and is non-toxic. It could thus be feared that the enzymes required for synthesis could be spread by horizontal genetic transfer, rendering possible the advent of scenario E. Phosphonoalanine should therefore be restricted to experimental scenarios A, B and D, in which the biosynthesis of xeno nucleosides is excluded.

The chemical series of iminodicarboxylate phosphoramidates i.e. P–N conjugates of the three compounds of structure $HN[(CH_2)mCO_2H][(CH_2)nCO_2H]$ m and n being 1 or 2, offers an opportunity for extending metabolism so as to encompass novel nucleic acid leaving groups. Iminodicarboxylates can be endogenously produced from or degraded to the common metabolites glycine, glyoxylate, beta-alanine and malonate semialdehyde through enzymatic reactions related to opine metabolism. Condensation of cyclotriphosphate with an iminodicarboxylate occurs spontaneously at appropriate concentrations and pH producing triphosphoiminodicarboxylate (TPI) [54,43]. Putative enzymes capable of accelerating this spontaneous reaction could possibly be found in the class of hydrolases that act on acid anhydrides, such as trimetaphosphatase (EC 3.6.1.2) [55]. Our study, however, showed that further condensation of triphosphoimidiacetate with the 5′ hydroxyl of the nucleoside deoxyadenosine did not proceed spontaneously under the various conditions tested [43]. Nevertheless, since this reaction features the nucleophilic attack of a phosphoanhydride by a primary alcohol as performed by many kinases, the systematic assay of such enzymes could unveil a biocatalyst marginally endowed with the desired activity.

In the long run, a fully integrated metabolic cycle disentangled from canonical nucleoside triphosphate could likely be assembled for reloading xeno nucleosides into activated substrates of a xeno polymerase.

7. Conclusion

The in vitro exploration of alternative leaving groups in synthetic nucleotides to be subjected to templated enzymatic polymerization has enabled the identification of numerous and unsuspected activated chemical constructs that can undergo condensation by DNA polymerases. Most of the assays focused on the substitution of the regular dNTPs by deoxynucleotides activated with pyrophosphate surrogates of the phosphoroamidate types. They unveiled a broad tolerance of polymerases toward such unnatural metabolic precursors of nucleic acids, even though incorporation was often limited to one to three nucleotides. There is little doubt that directed evolution will afford mutant polymerases processively catalysing unlimited incorporation of surrogate dNTPs lacking a pyrophosphate moiety. Such artificial phylogenies of polymerases will be pertinent for addressing profound scientific questions such as the metabolic conditions for emergence of informational polymers as well as technological opportunities such as the tightly controlled propagation of additional classes of nucleic acids, thus extending the central dogma of molecular biology for resuming molecular evolution without endangering natural species.

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